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THERMAL CYCLER TEMPERATURE VARIATION AND ITS EFFECT ON THE POLYMERASE CHAIN REACTION

by

D.E. Bader

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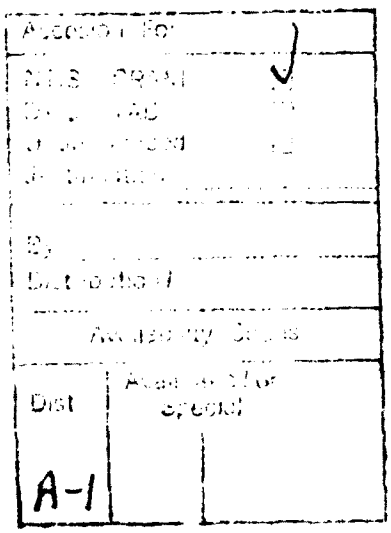

ABSTRACT

This study was undertaken to investigate the source of variation in Polymerase Chain Reaction (PCR) amplification assays that we have encountered periodically in our studies. Two approaches, namely, PCR/agarose gel analysis and thermal probe analysis, were used in this investigation. PCR/agarose gel analysis demonstrated random variation in the quantity and quality of amplified product from well to well, both within and between trials. Several modifications to the procedure did not eliminate variability. Thermal probe analysis indicated significant well to well temperature variability among certain wells or groups of wells within in trial ($p=0.01$) and significant temperature variability among certain well positions from one trial to another ($p=0.05$). Thermal probe analysis also indicated large differences between the programmed setpoint temperatures and the actual temperatures inside the tubes for all three PCR events at the beginning of the soak period and two of three PCR events at the end of the soak period. The data from this investigation and other studies leads to the conclusion that the source of variability in PCR amplification efficiency we have experienced is most likely due to the inherent inability of our thermal cycler to maintain consistent temperature homogeneity across the heating block during PCR amplification reactions. It is advisable that replicate samples for amplification be prepared since a particular well cannot be expected to provide consistent results from well to well within a trial or from one trial to the next.

RÉSUMÉ

La présente étude a été entreprise pour déterminer la source de la variabilité que nous avons observée dans les essais d'amplification par la réaction en chaîne par la polymérase (PCR) au cours de nos travaux. Deux approches, la PCR/analyse sur gel d'agarose et l'analyse par sonde thermique, ont été utilisées au cours de la présente étude. La PCR/analyse sur gel d'agarose a mis en évidence une variation aléatoire de la quantité et de la qualité des produits amplifiés, d'un puits à l'autre, aussi bien dans un même essai qu'entre des essais différents. L'analyse par sonde thermique a indiqué une variabilité interpuits substantielle dans certains puits ou groupes de puits dans un même essai ($p = 0,01$) et une variabilité substantielle de la température entre certaines positions de puits d'un essai à un autre ($p = 0,05$). L'analyse par sonde thermique a également indiqué des différences importantes entre les températures de consigne programmées et les températures réelles à l'intérieur des tubes au cours des trois événements de PCR au début de la période d'imprégnation et au cours de deux des trois événements de PCR à la fin de la période d'imprégnation. Les données de cette étude et d'autres études nous amènent à conclure que la variabilité de l'efficacité de l'amplification par PCR que nous avons rencontrée est fort probablement attribuable à l'incapacité inhérente de notre cycleur thermique de maintenir une température homogène dans la totalité du bloc de chauffage durant les réactions en chaîne par polymérase. Il est recommandé de préparer des échantillons en double pour l'amplification étant donné que l'on peut s'attendre qu'un puits particulier donne des résultats uniformes par rapport aux autres puits au cours d'un même essai ou d'un essai à un autre.

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INTRODUCTION

The Polymerase Chain Reaction (PCR) is a technique which is used to amplify specific nucleic acid sequences *in vitro* and is used in a wide number of applications (for a review see [1]). The principle behind the PCR amplification reaction involves the repeated cycling of three molecular events: (a) denaturation of template and primer nucleic acid sequences into single stranded form; (b) hybridization of the primer sequences to the template; (c) extension of the primer sequence by a heat stable DNA polymerase enzyme which incorporates the appropriate complementary nucleotide onto the 3'end of the primer sequence. These three molecular events are carried out under very controlled conditions of time and temperature which are dependent upon several factors such as nature and purity of the template, nature of the PCR primers, Mg^{+2} concentration, etc. Typically, the denaturation step is carried out at 94°C for 1 min; followed by the hybridization step at 37°C for 2 min; followed by the extension step at 72°C for 3 min. One cycle includes each of these three events and each cycle is repeated 20-40 times. Time, temperature and number of cycles, are usually determined empirically to find the optimum values for a given PCR reaction. PCR can be carried out using two temperatures rather than three if the hybridization and extension steps can be effectively performed at temperatures near the extension temperature. The success of the PCR reaction is, to a large extent, dependent upon the ability to provide precise and accurate control of time and temperature for each of these molecular events. Various commercial suppliers have developed DNA thermal cyclers which are designed specifically to control time and temperature parameters for PCR in an automated fashion.

We have used PCR in our research to amplify specific regions of target nucleic acid sequences for use in gene probe hybridization assays and gene cloning experiments. When performing PCR amplifications, we have noticed, at times, variations in the amount of amplified product from well to well or from one trial to another. It has been stated that a variation of a factor of 3-5 is normal in PCR and can be predicted theoretically [2]. However, we have experienced variations of up to 20 fold or greater and we have also encountered amplification failures for samples that had, at other times, generated strong positive signals. Weak amplification signals are not of a major concern for those analyses in which a qualitative result is required, for example, when screening clones for the presence of a cloned insert or in cases where large amounts of amplified product are not required for subsequent analysis or manipulation, for example, sequencing or cloning. However, it is more of a concern when large quantities of material are required for subsequent analyses, for example, when preparing large amounts of material for use as nucleic acid probes. Amplification failures pose more of a concern than weak amplifications, both qualitatively and quantitatively, since they can lead to false conclusions. Because PCR is becoming more widely used in our research, it would be advantageous to understand the cause of this variation especially for those cases in which amplification failures occur. Consequently, this study was initiated to investigate the source of variability in our PCR amplifications. Two approaches were used in this study. The first approach involved carrying out PCR on identical samples (prepared from a master PCR reaction mixture) placed in reaction tubes in all 48 well positions of the thermal cycler heating block and analyzing the results by horizontal agarose gel analysis. The second approach involved using a thermal probe to measure the temperature inside the reaction tube for eight randomly selected well positions.

MATERIALS AND METHODS

DNA Thermal Cycler

The DNA thermal cycler used in this study was a Perkin-Elmer Cetus DNA thermal cycler (serial no. P3440). The block sample capacity for this model is 48 x 0.5 mL microcentrifuge tubes. According to the manufacturer's specifications, the temperature accuracy (measured directly in the wells) is +/- 3°C in the range of 4-32°C and +/-1°C in the range of 33-100°C .

PCR Amplification

A master mix of PCR reaction components was made using components from the Perkin Elmer Cetus Geneamp™ DNA Amplification Reagent Kit. It consisted of the following components in the order listed: 588.5 μL sterile 3x distilled water, 110 μL of 10x reaction buffer (100 mM Tris-HCl; 500 mM KCl; 15 mM $MgCl_2$; 0.1% (w/v) gelatin), 176 μL of 10x dATP:dCTP:dGTP:dTTP mix (1.25 mM each), 55 μL of control primer 1 (20 μM), 55 μL of control primer 2 (20 μM), 5.5 μL of Amplitaq™ and 110 μL of lambda phage DNA template (0.1 ng/μL). Twenty μL of the master mix were aliquotted carefully into forty eight 0.5 mL microfuge tubes. Two types of tubes were used in this investigation: (1) GeneAmp™ reaction tubes (Perkin-Elmer Cetus, catalog no. N801-0180); (2) siliconized Diamed tubes (Diamed, catalog no. PRE050S-N). All microfuge tubes were sterilized by autoclaving for 20 min (15 psi, 240°C) prior to use. Two drops of light white mineral oil (Sigma, d=0.84 g/mL) were layered onto the reaction mixture in each tube to prevent evaporation/refluxing. The 48 replic- samples were placed into the thermal cycler

(pre-warmed for minimum of 0.5h) and the following temperature cycling profile was used: 94°C for 1 min; 37°C for 2 min; 72°C for 3 min. This cycle was repeated for a total of 30 cycles, followed by 72°C incubation for 7 min to allow for the extension of incomplete strands. All other modifications to this procedure are described in the results section. Two μL of 10x stop buffer (0.1% bromphenol blue; 1% SDS; 0.1 M EDTA (pH 8.0); 50% glycerol) were added to each sample, mixed by vortexing and then centrifuged briefly (30 sec) in an Eppendorf microfuge at 14,000g. The samples were stored in -20°C freezer until analyzed by horizontal agarose gel electrophoresis at which time all samples were thawed, mixed by vortexing and centrifuged for 30 sec at 14,000g. The PCR primers used in this study are designed to amplify a nucleic acid sequence region of 500 base pairs (bp).

Horizontal Agarose Gel Analysis

PCR samples were analyzed on 1% agarose gels (BRL). Two double comb gels (28 sample wells per gel) were prepared by melting 0.75 g of agarose in 75 mL of 1x TAE buffer (40 mM Tris pH 8.2; 20 mM sodium acetate; 1 mM EDTA (pH 8.0)). The molten agarose was cooled to 65°C and then 4 μL of ethidium bromide (10 mg/mL) was added to give a final concentration of 0.5 μg/mL. Two 14-well gel combs were placed in a 14 x 10 cm gel boat (one above the other). The molten agarose was poured into the gel boat and allowed to harden for approximately 1 hour. The gel was placed in a horizontal electrophoresis tray and submerged under 1x TAE buffer. Ten μL of each sample (bottom layer) were drawn through the upper oil layer using a Gilson pipettmen™ and then loaded into the well. Ten μL of 0.1 μg/μL HindIII digested lambda phage molecular weight standards were loaded into the outermost wells. The gel was run at 50 V for 1.5 to 3 hours. The DNA was

visualized by exposure to UV light and a photograph of the gel was taken using Polaroid Type 57 film (f-stop=F8, exposure=0.5 sec, camera height=56 cm).

Thermal Probe Analysis

One hundred μL of H_2O were placed into each of 48 Diamed microfuge tubes and then overlaid with two drops of light mineral oil (Sigma, density= 0.84g/mL). Prior to placing the tubes into the wells, the thermal cycler was pre-warmed for a minimum of 0.5h, followed by running the auto-tune diagnostic program (diagnostic program #6) according to the manufacturer's instructions. One drop of heavy mineral oil (Sigma, density = 0.88 g/mL) was placed in each well to enhance thermal conductivity between the well and the tube and then each tube was placed into one of 48 wells. The temperature inside the reaction tube was measured by inserting a temperature probe (Omega Engineering Inc., model 412B-T) through a hole in the top of the microfuge tube cap. Thermal probe analysis was performed on eight of forty-eight well positions on the thermal cycler block. These positions were selected at random and included well positions A3, A5, B6, B8, C8, D1, D2 and E8 (Figure 1). The temperature profile used in this experiment included the following cycle: 94°C for 1 min; 37°C for 1 min; 72°C for 1 min. Temperature readings were taken at 15 sec intervals during the 1 min soak period, beginning at the time in which the thermal cycler sensors indicated that the heating block had reached the setpoint temperature (time = 0 sec). Six cycles were measured for each of the 8 well positions which constituted one trial. Two trials were carried out on two separate days.

RESULTS

Method 1: PCR Amplification /Horizontal Agarose Gel Analysis

Experiment 1

The PCR reaction was carried out on identical samples placed in all 48 well positions of the thermal cycler heating block according to the procedure outlined in "Methods and Materials". Briefly, twenty μL of a master mix of PCR reaction components were aliquotted carefully into forty eight 0.5 mL siliconized Diamed microfuge tubes. The 48 replicate tubes were placed into all wells of the thermal cycler (pre-warmed for 0.5h). A temperature cycling profile of 94°C (1 min); 37°C (2 min); 72°C (3 min) for 30 cycles, followed by 72°C incubation for 7 min, was performed and the samples were then analyzed by horizontal agarose gel electrophoresis.

The results of the horizontal agarose gel analysis from this experiment are presented in Figures 2a and 2b. There was noticeable variation in the quantity of the 500 bp product among the 48 samples as evidenced by differences in band intensities. Well position C8 generated a very weak product band equivalent to about 50 ng of DNA (determined by comparison to the intensity of the 2322 mwt band of the λ-Hind III markers which is equivalent to about 50 ng) while several other well positions (D1, D3, D4, D6, D8, E3, E5, E7, E8, F4, F5, and F8) generated strong bands equivalent to about 1000 ng or more. This variation represented at least a 20 fold difference. There were two well positions which did not generate any visible 500 bp product (C6 and C7) and were considered to be amplification failures. Diffuse

banding below the 500 bp product band was evident in many of the samples which also varied in intensity. This diffuse banding was most likely due to the formation of primer-oligomers which are formed as a consequence of the two PCR primers having GG/CC overlapping 3' ends.

In order to ensure that the cycler was working properly prior to running further PCR amplifications, pre-programmed diagnostic programs were performed according to the instruction manual. These diagnostic programs included the heater test, the chiller test, the sensor test and the auto-tune initialize test. All diagnostic tests were found to be within the manufacturer's limits and therefore found to be acceptable. The auto-tune initialize test is of particular importance. According to the manufacturer, this test should be performed whenever poor temperature control is noticed. Consequently, this experiment was repeated following execution of the auto-tune program (Experiment 2).

Experiment 2

Figures 3a and 3b show the results from the first trial. Forty seven of forty eight wells (98%) generated very intense 500 bp product bands of nearly equal intensity with one well position (B2) generating slightly less. There were also no amplification failures for this trial. These results indicated very good reproducibility among the 48 wells which suggested that executing the auto-tune program prior to amplification, reduced well to well variability. However, when this experiment was repeated (Figures 4a and 4b), obvious variation in amplification efficiency was again evident as judged by variation in intensity of the bands and by the absence of amplified product in two of the wells (D3 and F7).

This data suggested that the well to well variability problem still existed and was not solved completely by running the auto-tune program. In addition, this data indicated trial to trial variation since trial 1 gave very little variation while trial 2 gave considerably more variation. A comparison of the results from experiment 1 and experiment 2 suggested that the variation was random in that a particular well which showed poor amplification or complete failure in Experiment 1 often showed good amplification in Experiment 2 and vice versa. For example, the two amplification failures seen in experiment 1 were generated in wells C6 and C7, while those in experiment 2 (trial 2) were generated in wells D3 and F7.

Consultation with the manufacturer resulted in two additional modifications to the procedure that were implemented in Experiment 3: (1) application of heavy oil inside the wells to enhance thermal conductivity between the well and the microfuge tube; (2) pre-heating the heating block to the initial denaturation temperature (94°C) prior to starting PCR to help reduce primer-oligomer amplification.

Experiment 3

The results from this experiment (Figures 5a and 5b) are similar to experiment 2, trial 1 in which 47 of 48 well positions (98%) generated similar, strong band intensities with one well position generating slightly less (well position A4). Again, there were no amplification failures and there was also a reduction in the amount of primer-oligomer product. This experiment suggested that the addition of the heavy oil in the wells, together with the pre-warming step (94°C) and auto-tuning prior to running PCR, may have helped to reduce well to well variability. When this

experiment was repeated (Figures 6a and 6b), the band intensities for most of the wells were similar indicating good reproducibility, however, the product band in well position F8 was completely missing. Well position F8 had not generated an amplification failure prior to this experiment (ie. previous amplification failures were seen in well positions C6, C7, D3 and F7) supporting the observation that the cause of this variation seems to be random.

Experiment 4

Experiment 4 was performed in identical fashion to experiment 3 except that microfuge tubes specifically designed for the Perkin-Elmer Cetus thermal cycler (GeneAmp™) were used in place of the Diamed tubes. In addition to being made by different manufacturers, these two tubes differed in that the Diamed tubes were siliconized while the GeneAmp™ tubes were not. The results of this experiment can be seen in Figures 7a and 7b. Again, there was obvious well to well variability with some well positions generating very intense bands (approx 1000 ng in each of well positions E4 through F8) versus well positions which generated weak bands (approx. 50 ng or less for well positions A1, B7 and C6) representing approximately a 20 fold difference in the amount of amplified product. These results suggested that the GeneAmp™ tubes did not resolve the variability problem experienced in previous experiments in which Diamed tubes were used.

Method 2: Thermal Probe Analysis

The thermal probe test was used to measure the performance of the thermal cycler by monitoring the actual temperature inside the reaction tube for eight

randomly selected well positions (Figure 1). The thermal cycler diagnostic tests (heater test, chiller test, sensor test and auto-tune initialize test) were performed prior to PCR and found to be within the manufacturer's suggested limits.

The results obtained from the thermal probe analysis are plotted in Figures 8, 9 and 10 for the three PCR events (denaturation, hybridization and extension, respectively). These figures demonstrate three important features. First, the general shape of the curves for all well positions in all three PCR events was asymptotic. This indicated that the temperature inside the tube at the beginning of the soak period was not the same as that measured at the end of the soak period. In most cases, the curves levelled off by the end of the 60 second soak period indicating that they had reached their final equilibrium temperatures. Second, the curves for all three PCR events indicated greater divergence at the beginning of the soak period than at the end. Third, there was an obvious difference between the setpoint temperature (the temperature programmed into the thermal cycler for each of the three PCR events) and the measured or actual temperature. For the hybridization event (Figure 9), some well positions reached the setpoint temperature of 37°C before the end of the 60 sec and actually fell below 37°C by the end of the 60 sec soak period, while other wells never reached 37°C even after 60 sec. For the extension and denaturation steps, none of the well positions reached their respective setpoint temperatures even after 60 seconds (Figures 8 and 10).

An analysis of variance (Student-Newman-Keuls Multiple Comparison test) was performed on the data for each of the eight well positions at both the beginning and end of the soak period for all three PCR events. Table I summarizes the results of this analysis with respect to those well positions or groups of wells which showed

significant differences at a 99% significance level (p=0.01). The analysis results indicated that for the denaturation event, there were 3 groups of wells which were significantly different from each other at the beginning of the soak period and 5 groups that were significantly different at the end of the soak period. The results for the hybridization event indicated that all 8 well positions were significantly different at the beginning of the soak period but only 6 out of 8 were significantly different at the end. Finally, the results for the extension event indicated 4 significantly different groups of wells at the beginning of the soak period and 3 at the end. There does appear to be a trend among this data in that well positions E8, D1 and D2 were found to exhibit mean temperatures further from the other wells (with respect to the setpoint temperature) for all three PCR events. Table II includes the mean temperatures for the wells or groups of wells that were determined to be significantly different in Table I. The temperature range (the mean temperature difference between those wells which generated the highest and the lowest mean temperatures) was found to be greater at the beginning of the soak period (3.4°C-denaturation; 7.6°C-hybridization; 4.1°C-extension) than at the end of the soak period (2.2°C-denaturation; 1.8°C-hybridization; 1.6°C-extension) for all three PCR events.

As mentioned previously, thermal probe analysis indicated that there was a difference between the setpoint temperature (the temperature programmed into the thermal cycler for one of the three PCR events) and the measured or actual temperature measured. Table III summarizes the lowest and highest temperature differences among those well positions or groups of wells which were shown to be significantly different (p=0.01). Again, for all three PCR events, the temperature variation was found to be greater at the beginning of the soak period (12-16°C-denaturation; 4.0-12.0°C-hybridization; 11.0-15.0°C-extension) than at the end of the

soak period (6-8°C-denaturation; +/-1.0°C-hybridization; 5-6°C-extension) for all three PCR events.

The extent of temperature variation for a particular well position from one trial to another was examined by performing statistical analysis on each well position with data from two trials. The results of this analysis are presented in Table IV. Most of the well positions showed a significant temperature difference (ΔT) between the two trials based on a 95% level of significance ($p=0.05$). The average temperature difference, among those wells which showed a significant difference, was greater at the beginning of the soak period than at the end for all three PCR steps, with the extension event showing the greatest temperature range. Well positions which exhibited ΔT values greater than the average included well E8 for the denaturation event; wells D1, D2, and E8 for the hybridization event; and wells C8, D2 and E8 for the extension event.

According to the manufacturer, poor temperature control can be a result of low or high line voltage problems. Performing the auto-tune program can help alleviate this problem but this was shown to be ineffective since the auto-tune program was performed prior to thermal probe analysis for all PCR runs and variability was still evident. Subsequently, a line noise suppressing ultra-isolator (Webster Instruments Ltd.) was connected to the thermal cycler and thermal probe analysis was performed using the same procedure as previously described to determine if the variability problem could be reduced. The results from this analysis (data not shown) indicated that temperature variability was worse than that obtained without the ultra-isolator.

DISCUSSION

PCR/horizontal agarose gel analysis gave a quantitative and qualitative indication of the PCR amplification efficiency for all 48 well positions of the heating block. The results indicated that there was variation in the amount of amplified product from well to well within a single trial and that the degree of variation ranged from minimal to large (20 fold differences), including some amplification failures. Trial to trial variation was evident from comparisons of band intensities from one trial to another, especially for those well positions which generated amplification failures. That is, wells which generated strong, intense bands in one trial, generated weaker bands or no bands in a repeated trial. The variation among the 48 well positions appeared to be random in that wells which generated strong signals in one experiment generated weak or no signals in others and vice versa. Large well to well variation and/or amplification failures that we observed in this analysis could not be attributed to differences in reaction component concentrations (or missing components in the case of amplification failures) from one reaction tube to another since all 48 tubes contained an aliquot from a master mixture. In addition, although possible, it is highly unlikely that large well to well variations and/or amplification failures could have arisen from pipetting errors when loading electrophoresis gels since the variation in many cases was so large that one would have had to make a 95% error in order to generate these differences (50 ng in one well and up to at least 1 μg in another represents about a 95% error). Therefore, variability must have arisen from other factors such as: (a) variation in thermal transfer between the heating block and the reaction tube due to variation in reaction mixture volumes or variation in reaction tube thickness, and/or (b) the inability of the thermal cycler to provide temperature homogeneity for every well position across the heating block.

If pipetting inaccuracies or variations in reaction tube thickness were responsible for large variations and/or amplification failures, one would expect this source of error to be random and thus one would expect to find a similar degree of variation among all six experimental trials. Our data does not reflect this hypothesis since half of the experiments showed very good reproducibility among the 48 wells while the other half showed obvious well to well variability. Therefore, it is more likely that large variations in amplification efficiency were a result of thermal cycler itself. A study by the University of Alberta [annex A-1,2] supports the argument that well to well variability is more likely a consequence of the inability of the thermal cycler to maintain temperature homogeneity from one well position to another than due to differences in reaction tubes or reaction volumes. They measured the temperature profile of 8 well positions simultaneously using eight solid-state temperature sensors (calibrated and matched to within 0.1°C) that were placed inside sealed individual 0.5 mL microfuge tubes containing 100 μL of mineral oil. The sensors measured the temperature at 0.5 second intervals for a program based on 92°C soak for 2 min, a rapid ramp to 55°C soak for 2 min and a rapid ramp to 72°C for a two min soak followed by a repeat of this cycle. This same analysis was performed on a three other thermal cyclers (Techne, Ericomp and Tyler Research Instruments). A comparison of the graphical results [Annex A-2] between the Tyler Research thermal cycler and the Perkin-Elmer Cetus cycler shows that the Perkin Elmer cycler exhibited greater well to well temperature variation among the 8 well positions tested. This same observation was evident for all well positions tested on the heating block (data not shown). If the temperature variability was a function of differences in reaction tube thickness or reaction mixture volume only, one might expect the Tyler Instrument cycler to exhibit temperature variation to the same extent as other cyclers. Since this is not the case, this study supports the idea that well to well

temperature variability may be more of a function of the thermal cycler itself rather than due to differences in reaction tube thickness or reaction volumes.

The results obtained from the thermal probe analysis in our study revealed that there were significant differences ($p=0.01$) among certain well positions or among groups of wells within a trial depending upon the PCR event and the time point during the soak period. Well to well temperature variation on average was found to be greater at the beginning of the soak cycle than at the end and was found to be outside the manufacturer's advertised range of 2°C for all three PCR setpoint temperatures (3.4°C-denaturation; 7.6°C-hybridization; 4.1°C-extension). Well to well temperature values at the end of the soak period were closer to o. within this range (2.2°C-denaturation; 1.8°C-hybridization; 1.6°C-extension). Whether or not the magnitude of these temperature differences are large enough to generate quantitative differences in the amount of amplified product is not known for certainty. However, it is conceivable that well positions which experience extreme temperature differences from others might generate quantitative and qualitative differences. For example, the data presented either in graphical form (Figures 8-10) or tabular form (Tables I, II and III), seem to indicate two populations of wells common to all three PCR events, namely, population A which includes well furthest from the setpoint temperature (D1, D2 and E8) and population B, which includes wells closer to the setpoint temperature (A3, A5, B6, B8 and C8). During the denaturation event, wells in population A experienced lower denaturation temperatures than those in population B which could be enough to generate reduced quantities of amplified products since incomplete denaturation of the target template DNA and/or the PCR product allows the DNA strands to re-anneal ("snapback") thereby reducing product yield or even generating amplification failures [3]. For the hybridization event, the

wells in population A did not reach the 37°C setpoint temperature before the end of the 60 second soak period, while most of the wells in the other population reached this temperature 20-40 sec into the soak period. Since higher annealing temperatures help to increase specificity [3], the wells in the first population may generate more specific product than those in population B. Finally, for the extension step, those wells in population A were further from the 72°C setpoint temperature than those wells in population B. This difference may be enough to generate unextended product molecules. In this study, an additional 7 minute extension step was included to promote completion of deficient product molecules.

Thermal probe analysis also indicated that there were statistically significant temperature differences ($p=0.05$) for certain well positions from one trial to another. Again these differences were greater at the beginning of the cycle (1.3°C-denaturation; 1.3°C-hybridization; and 2.0°C-extension) than at the end (0.7°C-denaturation; 0.2°C-hybridization; and 0.9°C-extension). Certain well positions seemed to generate larger temperature differences than others especially at the beginning of the soak period for different PCR events. Those well positions which were found to be above the mean ΔT at the beginning of the soak period included well E8 for the denaturation event; wells D1, D2, and E8 for the hybridization event; and wells C8, D2 and E8 for the extension event. It is interesting to note that wells D1, D2 and E8 were also found to generate the greatest well to well temperature variability in this study.

Thermal probe analysis showed that the actual equilibrium temperatures measured within the reaction tubes are on average 6-8°C and 4-6°C lower than the setpoint temperatures for the denaturation step (94°C) and extension step (72°C),

respectively. The equilibrium temperatures for the hybridization step were considerably closer to the setpoint temperature (+/-1°C). Overall amplification efficiency may be increased by raising the set temperatures for the denaturation and extension steps to accommodate these differences. In addition, increasing the holding time period to ensure that the final temperatures are maintained for the intended soak period, may increase the quality and quantity of amplified products.

This study was not designed to correlate temperature variability with amplification efficiency directly since these two parameters were investigated separately. However, the results from this study, along with other studies [annex A-1,2], indicates that there is well to well and trial to trial variability in PCR efficiency and that this variability is most likely due to the inability of our thermal cycler to maintain temperature homogeneity for different well positions in the heating block. It should be noted that the variability problem evident in this investigation utilized PCR primer sequences that matched the target DNA perfectly. The variability problem could be even more serious for primer/target sequences which contain mismatches.

It is advisable that when performing PCR amplification assays using our thermal cycler, one should prepare multiple samples for amplification since a particular well cannot be expected to provide consistent results from one well to the next within a trial or from one trial to the next. The auto-tune program should be run prior to executing PCR and heavy oil should be placed into the wells to enhance thermal conductivity since the "best" results were obtained under these conditions. The heating block should be pre-heated to the denaturation temperature prior to placing tubes inside the wells in order to minimize non-specific priming (eg. primer

oligomer artifacts). There does not appear to be any advantage to connecting a line voltage regulator to the thermal cycler and in fact, this may increase variability. Because thermal probe analysis showed that the actual temperatures within the reaction tubes are on average 6-8°C and 5-6°C lower than the setpoint temperatures for the denaturation step (94°C) and extension step (72°C), respectively, the quantity and quality of PCR amplified products might be increased by raising the set temperatures to accommodate these differences. Similarly, increasing the holding time period to ensure that the final temperatures are maintained for the intended soak period, may increase the quality and quantity of amplified products, since many of the well positions did not reach their equilibrium temperatures until 30-60 sec after the thermal cycler initiated the soak period countdown.

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Table I

Summary of Individual Well Positions or Groups of Wells Exhibiting Statistically Significant Temperature Differences

Denaturation (94°C)		Hybridization (37°C)		Extension (72°C)	
0 sec	60 sec	0 sec	60 sec	0 sec	60 sec
E8	E8,D1	B8	B8	E8	E8,D1
D1,D2	D2	C8	C8	D2,D1	D2
C8,A5, B8,B6, A3	A5,C8	B6	B6,A5	B8	B8,A5, C8,A3, B6
	A3,B8	A5	A3	C8,B6, A3,A5	
	B6	A3	E8		
		E8	D1		
		D1	D2		
		D2			

This table summarizes well positions which were found to be significantly different from each other at the beginning and the end of a 60 sec soak period for each of the three PCR events following an analysis of variance using the Student-Newman-Keuls' Multiple Comparison test. The analysis was based on six measurements for each well with a significance level of 99% ($p=0.01$). Well positions separated by a comma are not considered to be significantly different. The wells positions are arranged in the table such that those well positions at the top of the table generated lower mean temperature values than those below them.

Table II

Mean Temperature Values of Individual Well Positions or Groups of Wells Exhibiting Statistically Significant Temperature Differences

Denaturation (94°C)		Hybridization (37°C)		Extension (72°C)	
0 sec	60 sec	0 sec	60 sec	0 sec	60 sec
78.15	85.70*	41.42	35.97	56.50	65.82*
79.14*	86.18	42.08	36.20	57.66*	66.20
81.53*	86.90*	43.03	36.38*	59.43	67.37*
	87.38*	44.50	36.82	60.58*	
	87.88	45.98	36.98		
		47.37	37.68		
		48.65	37.80		
		48.98			
3.4	2.2	7.6	1.8	4.1	1.6

This table contains the mean temperatures (°C) for those well positions which were found to be significantly different from each other following an analysis of variance using the Student-Newman-Keuls' Multiple Comparison test (see Table I). For those cases in which the statistical analysis indicated no significant differences between two or more well positions, the mean temperatures for these well positions were averaged and are indicated by an asterisk. The values across the bottom row represent the temperature difference (°C) between wells which generated the highest and lowest mean temperature values.

Table III

Minimum and Maximum Temperature Differences Between the Setpoint Temperature and Measured Temperatures for Significantly Different Well Positions

	Beginning of Soak (t = 0 sec)	End of Soak (t = 60 sec)
Denaturation (94°C)	12.5 - 15.9 (below)	6.1 - 8.3 (below)
Hybridization (37°C)	4.4 - 12.0 (above)	1.0 (below) - 0.8 (above)
Extension (72°C)	11.4 - 15.5 (below)	4.6 - 6.2 (below)

The temperature values (°C) in the table represent the minimum and maximum temperature differences between the setpoint temperature and the actual measured temperature for those well positions which showed a significant difference (see Table I and II). The direction of this difference (above or below the setpoint temperature) is indicated in brackets.

Table IV

Table of Significant Temperature Differences Between Two Trials

	Denaturation (94°C)		Hybridization (37°C)		Extension (72°C)	
well	0 sec ΔT (°C)	60 sec ΔT (°C)	0 sec ΔT (°C)	60 sec ΔT (°C)	0 sec ΔT (°C)	60 sec ΔT (°C)
A3	0.5	0.5	0.7	-	1.7	0.8
A5	-	0.5	0.6	-	0.7	-
B6	-	1.4	-	0.3	-	1.0
B8	-	0.3	1.0	-	1.9	0.4
C8	1.3	0.6	0.9	0.1	2.2	0.9
D1	1.3	0.8	1.7	-	1.9	1.2
D2	1.2	0.6	2.3	0.2	2.7	0.9
E8	2.3	1.1	2.0	-	2.6	1.4
Avg	1.32	0.72	1.31	0.20	1.96	0.94
Std dev	0.64	0.36	0.68	0.10	0.67	0.32

A statistical T-test was run on a total of 6 readings for each well position for each of two trials using the statistics program Systat version 4.0. A significance level of 95% ($p < 0.05$) was chosen as the cut-off to eliminate data. If the T-test gave p-values greater than 0.05, the sample variances were not considered to be significantly different (indicated by "-") and were not included for calculation of the average or standard deviation values.

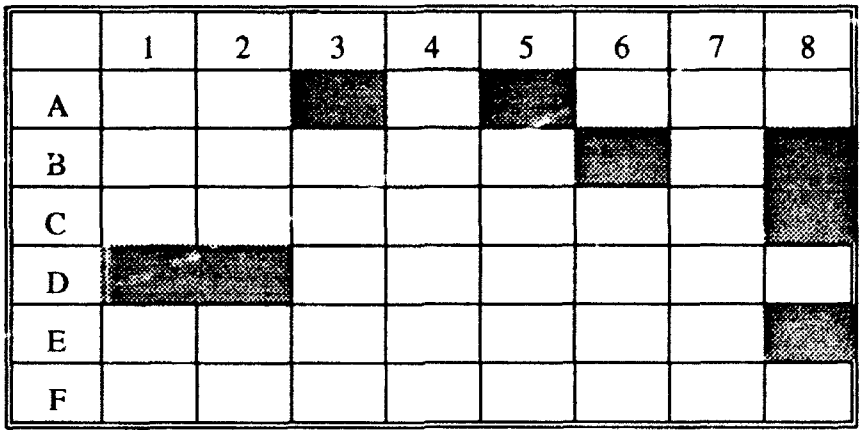


Figure 1

Schematic Diagram Depicting the Location of Eight Randomly Selected Thermal Cycler Heating Block Well Positions (shaded) Used in the Thermal Probe Analysis Experiment.

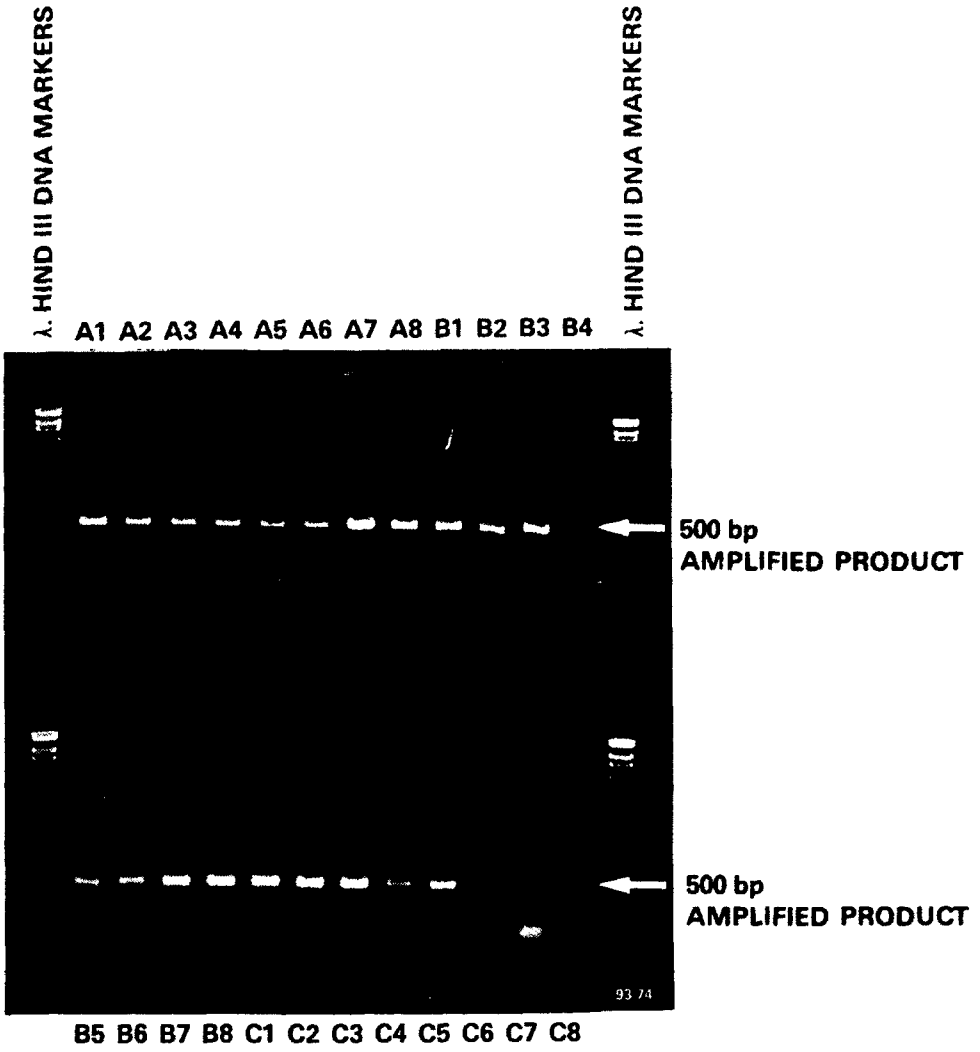


Figure 2a

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 1. Conditions include using 0.5 μL Diamed microfuge tubes (see "Methods and Materials" and "Results" section for more details). **Top row** = heating block well positions A1 through B4, **Bottom row** = heating block well positions B5 through C8. Hind III digested lambda DNA mwt markers (1.0 μg) are in outermost lane(s) for Figures 2a through 7b. The molecular weights of the marker bands from top to bottom are 23130, 9416, 6682, 4361, 2322, 2027, 564, and 123 bp (lower molecular weight bands may not always be visible).

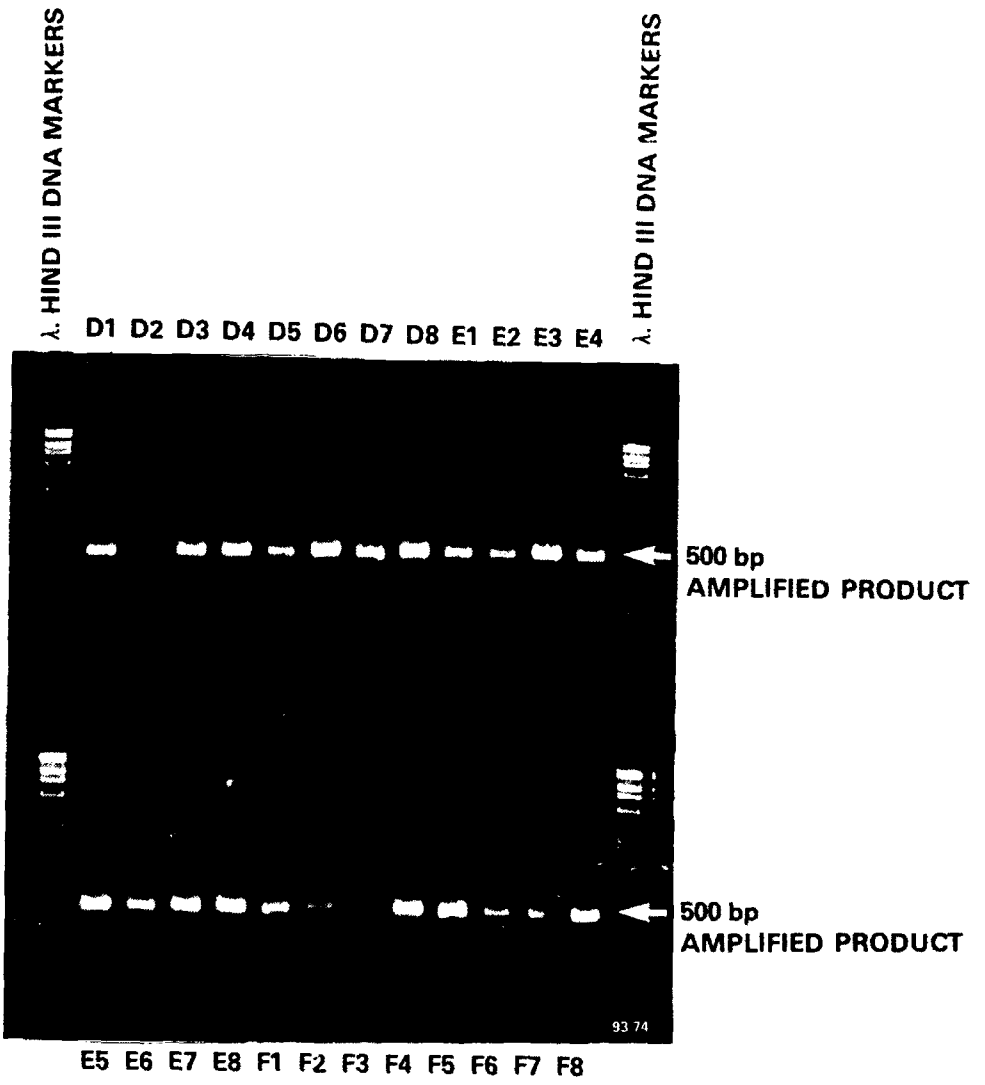


Figure 2b

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 1. Same as Figure 2a except: **Top row** = heating block well positions D1 through E4, **Bottom row** = heating block well positions E5 through F8.

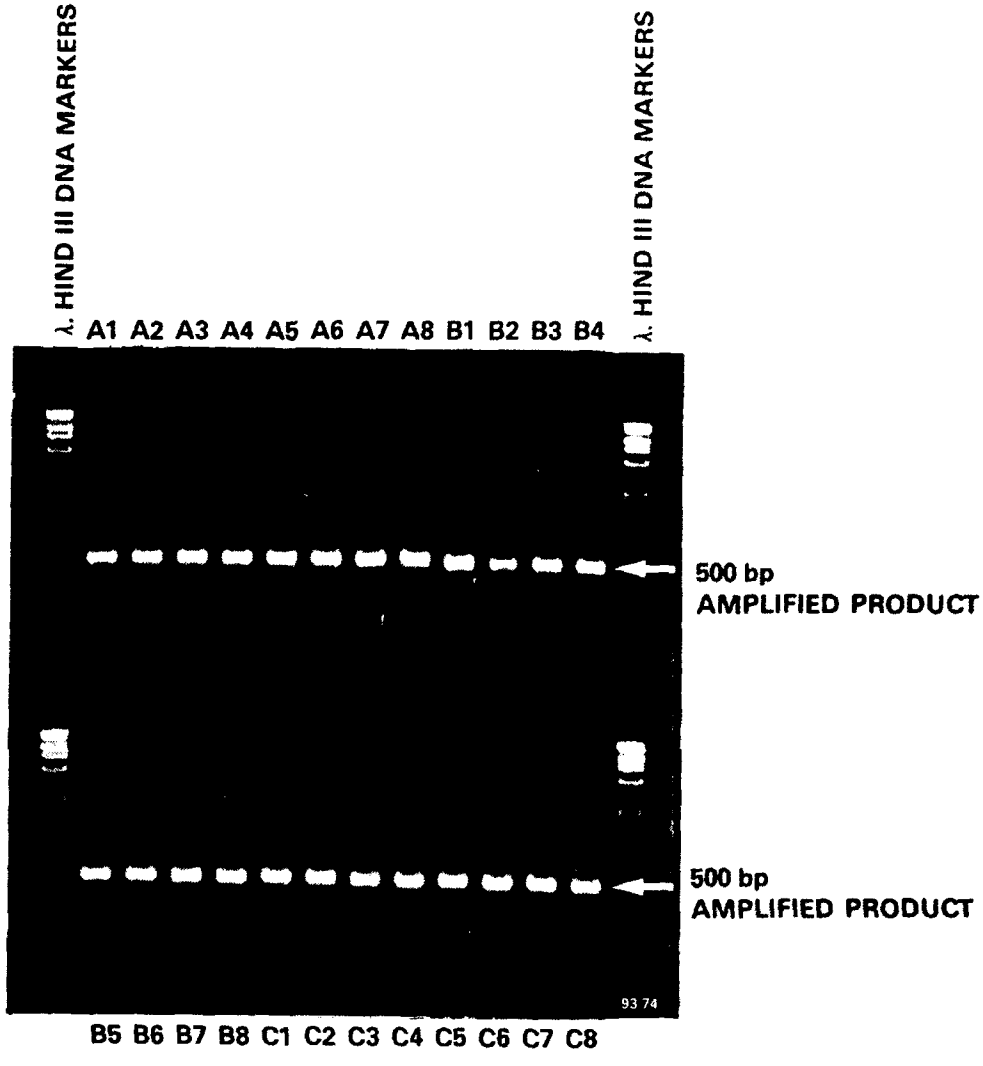


Figure 3a

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 2, Trial 1. Conditions include using 0.5μL Diamed microfuge tubes and the auto-tune test. **Top row** = heating block well positions A1 through B4, **Bottom row** = heating block well positions B5 through C8.

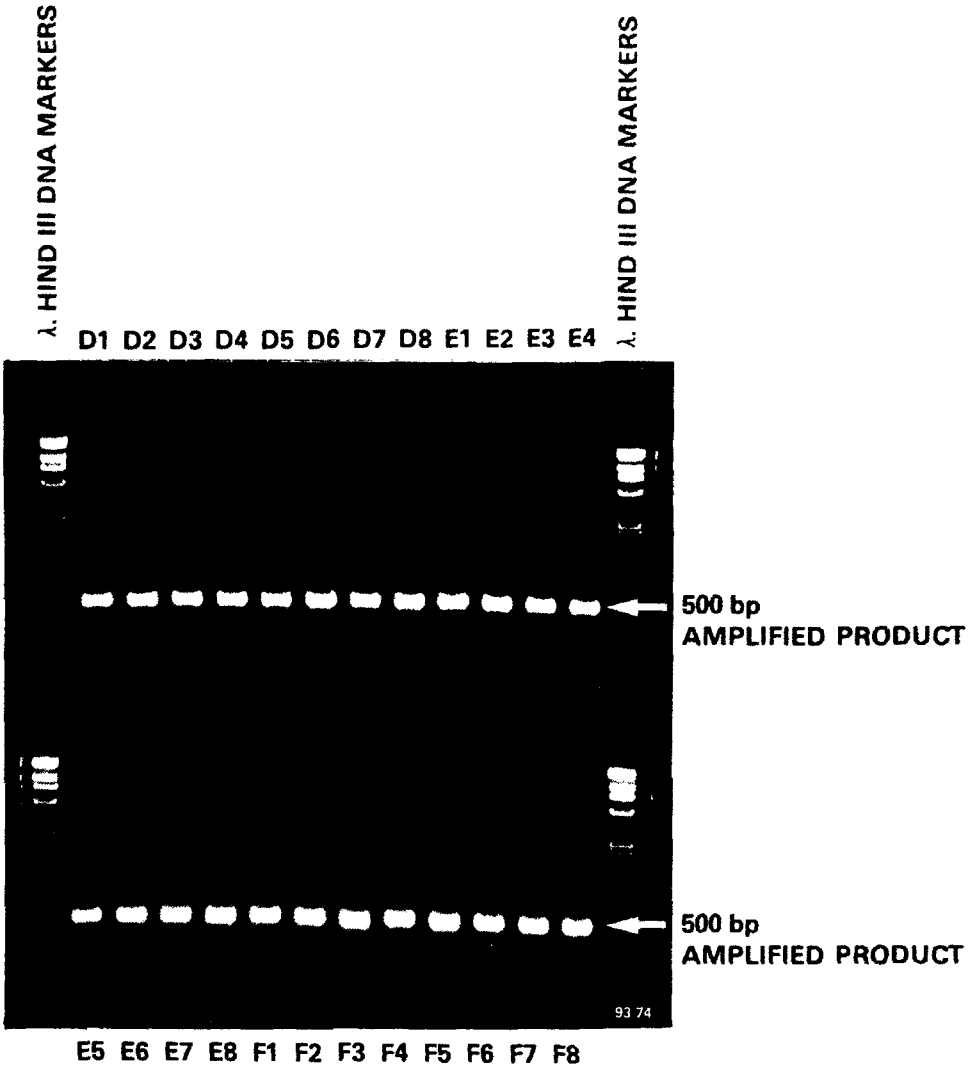


Figure 3b

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 2, Trial 1. Same as Figure 3a except: **Top row** = heating block well positions D1 through E4, **Bottom row** = heating block well positions E5 through F8.

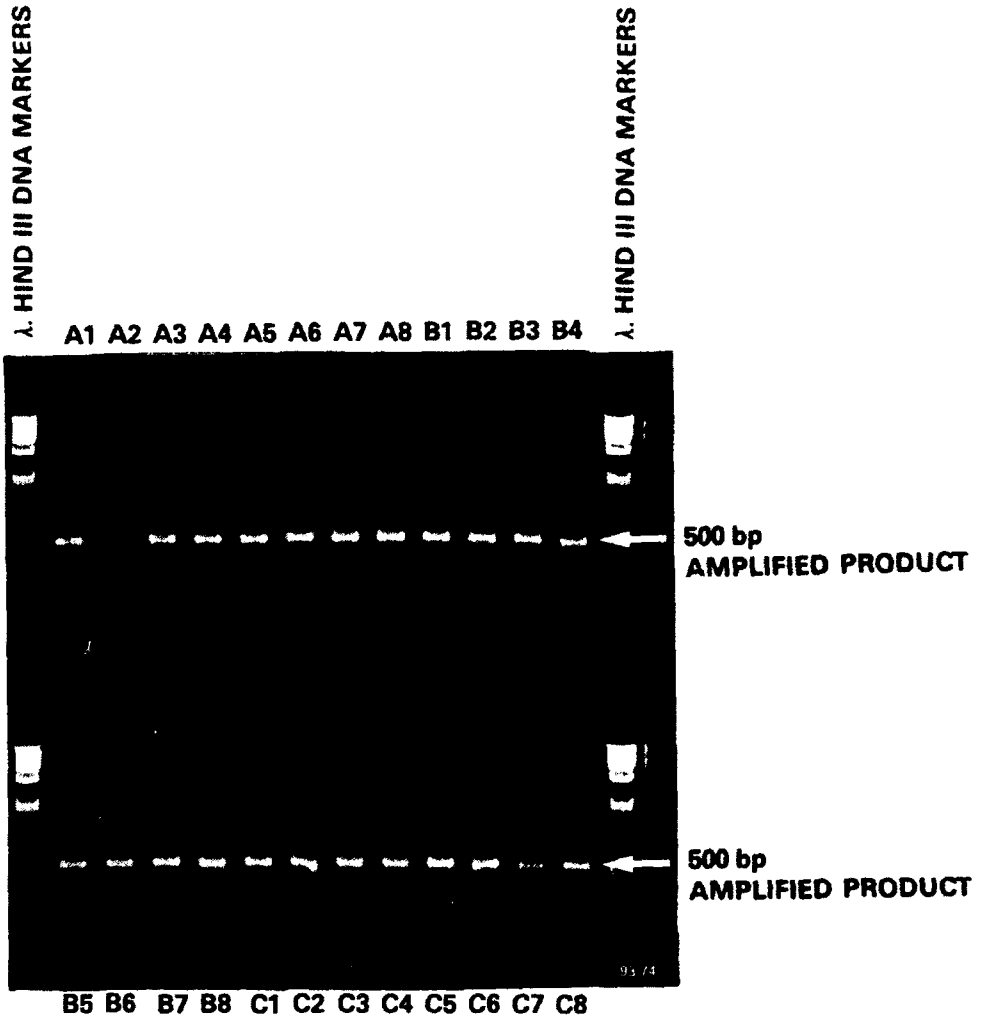


Figure 4a

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 2, Trial 2. Conditions include using 0.5 μL Diamed microfuge tubes and the auto-tune test. **Top row** = heating block well positions A1 through B4, **Bottom row** = heating block well positions B5 through C8.

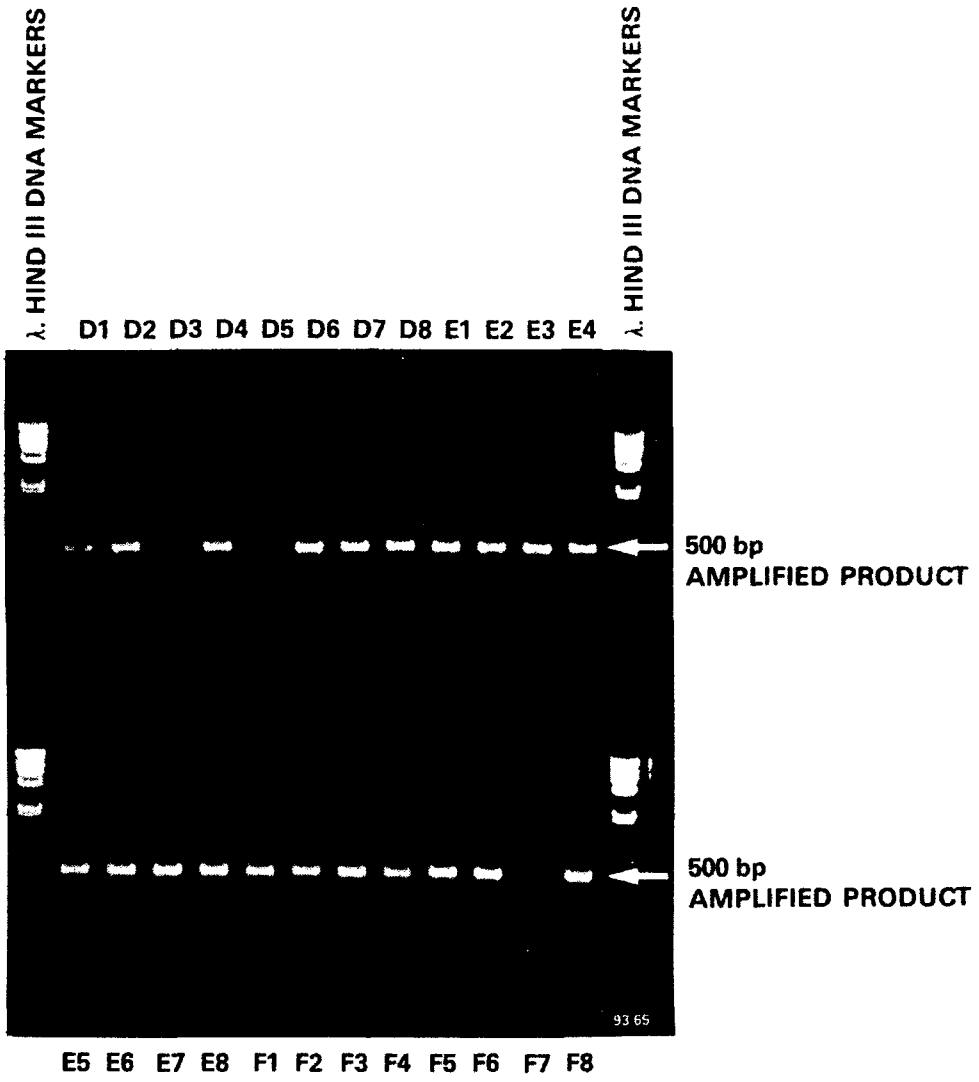


Figure 4b

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 2, Trial 2. Same as Figure 4a except: **Top row** = heating block well positions D1 through E4, **Bottom row** = heating block well positions E5 through F8.

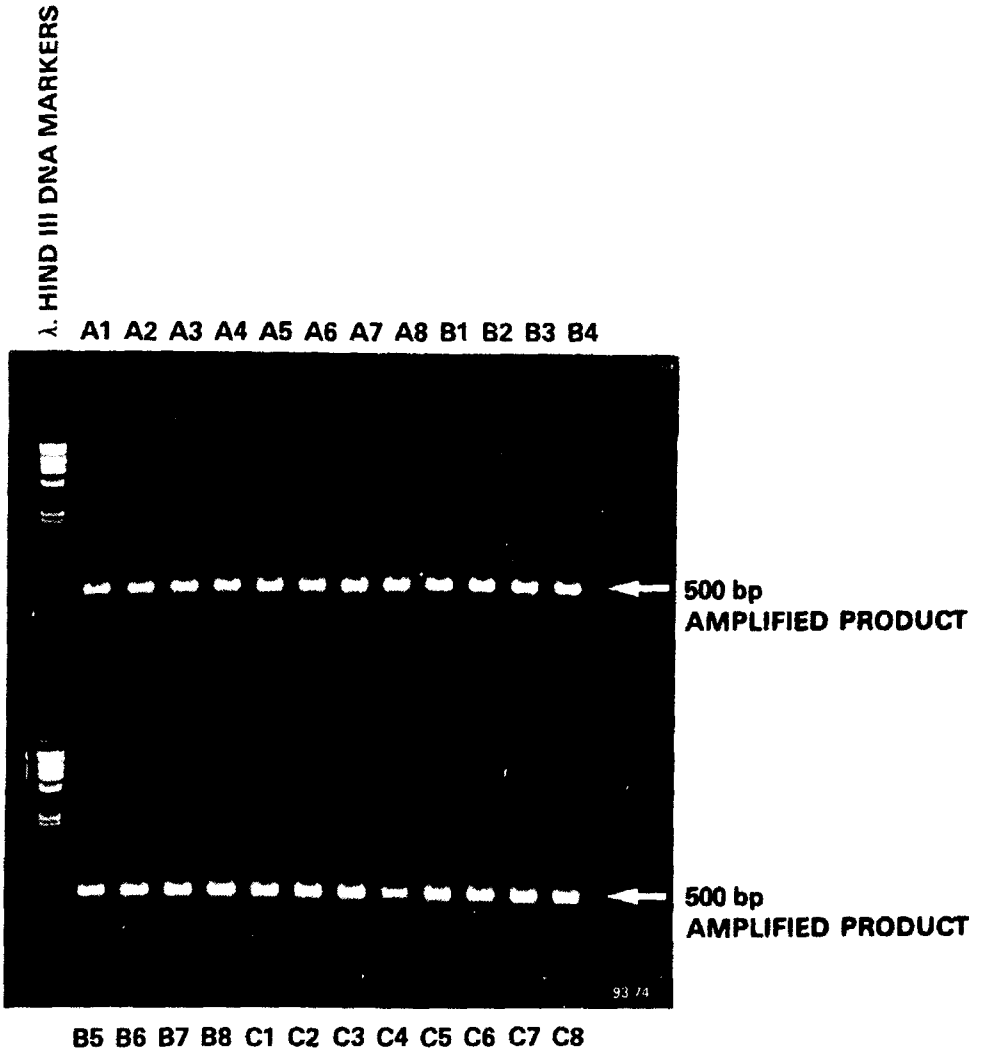


Figure 5a

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 3, Trial 1. Conditions include using 0.5 μL Diamed microfuge tubes, auto-tune test and heavy oil/94°C start. **Top row** = heating block well positions A1 through B4, **Bottom row** = heating block well positions B5 through C8.

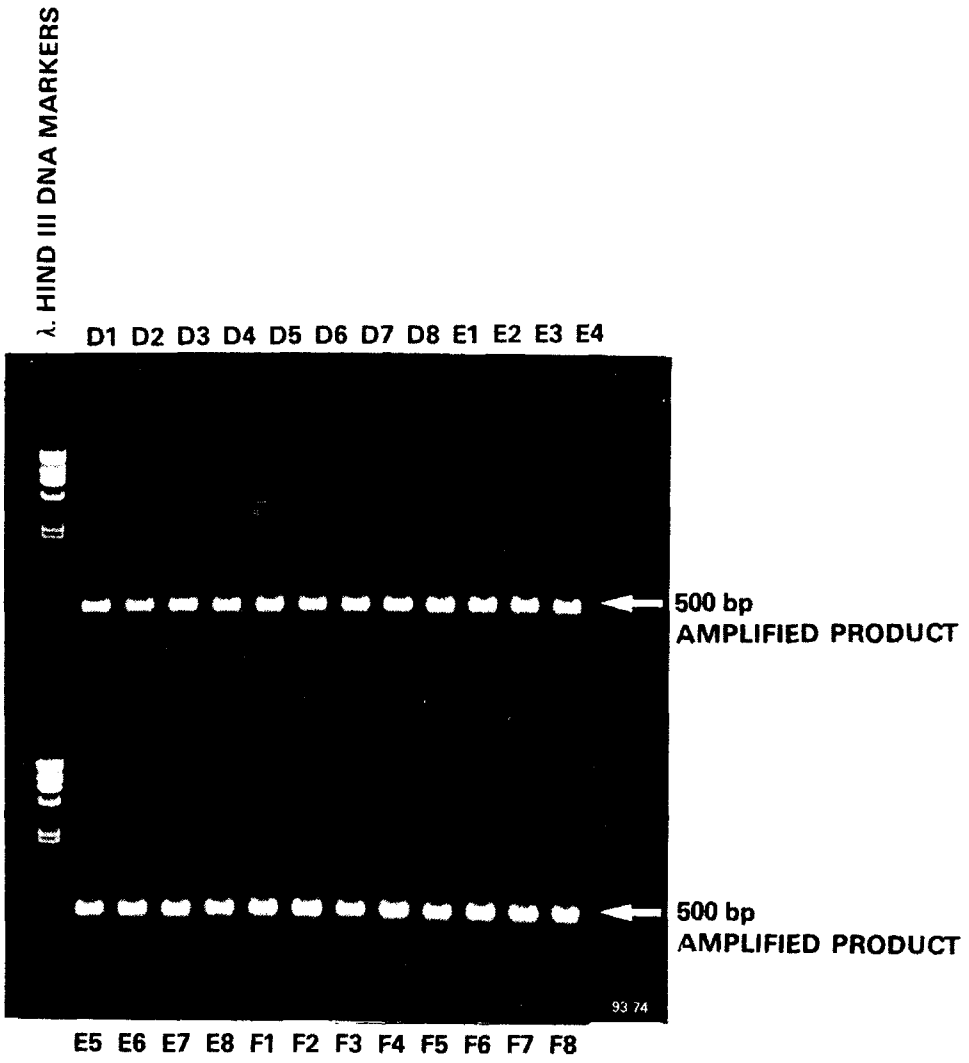


Figure 5b

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 3, Trial 1. Same as Figure 5a except: **Top row** = heating block well positions D1 through E4, **Bottom row** = heating block well positions E5 through F8.

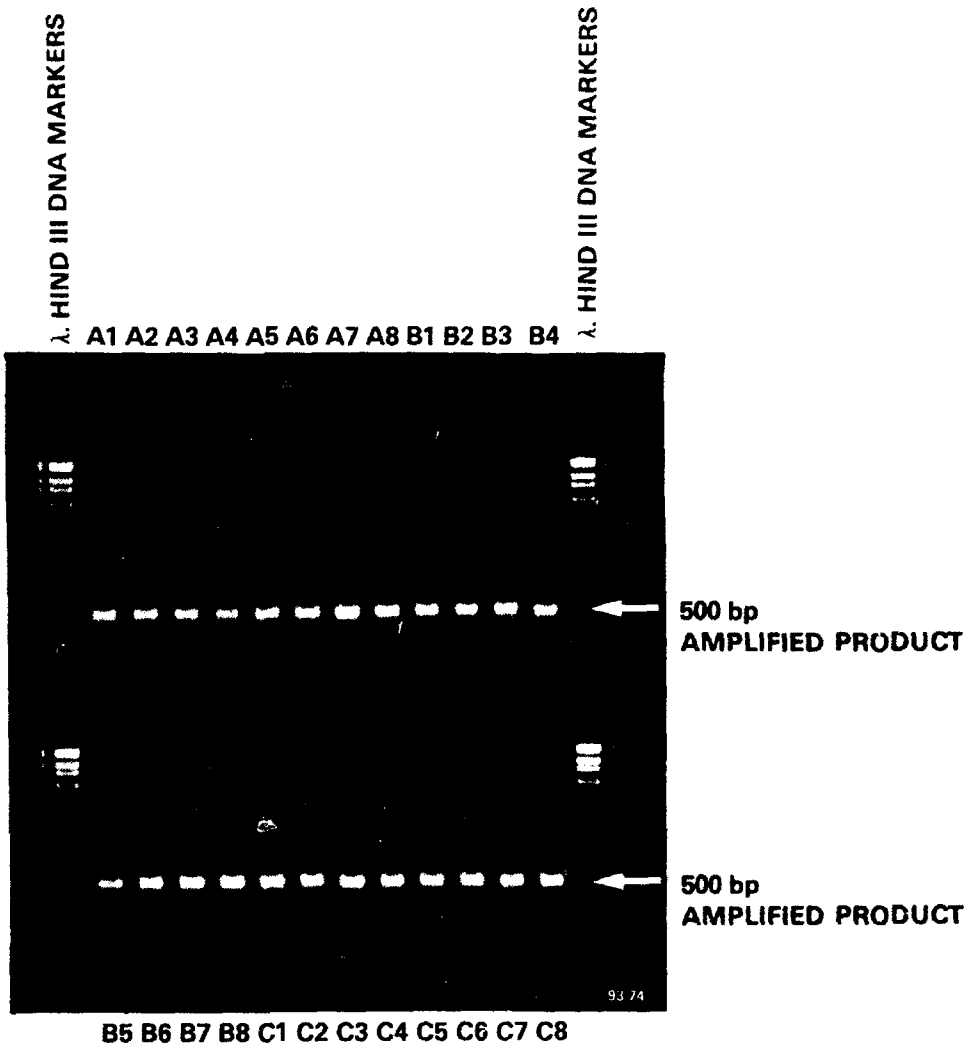


Figure 6a

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 3, Trial 2. Conditions include using 0.5 μL Diamed microfuge tubes, auto-tune test and heavy oil/94°C start. **Top row** = heating block well positions A1 through B4, **Bottom row** = heating block well positions B5 through C8.

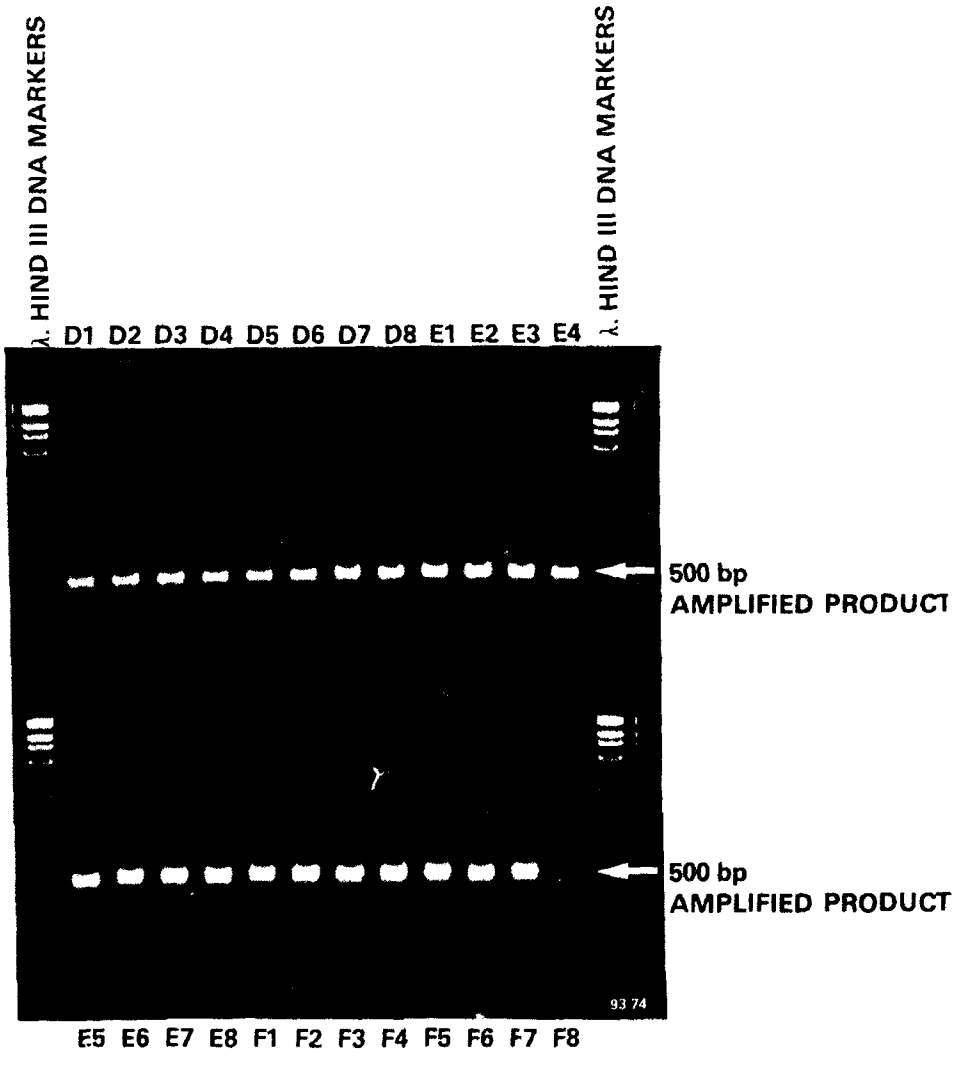


Figure 6b

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 3, Trial 2. Same as Figure 6a except: **Top row** = heating block well positions D1 through E4, **Bottom row** = heating block well positions E5 through F8.

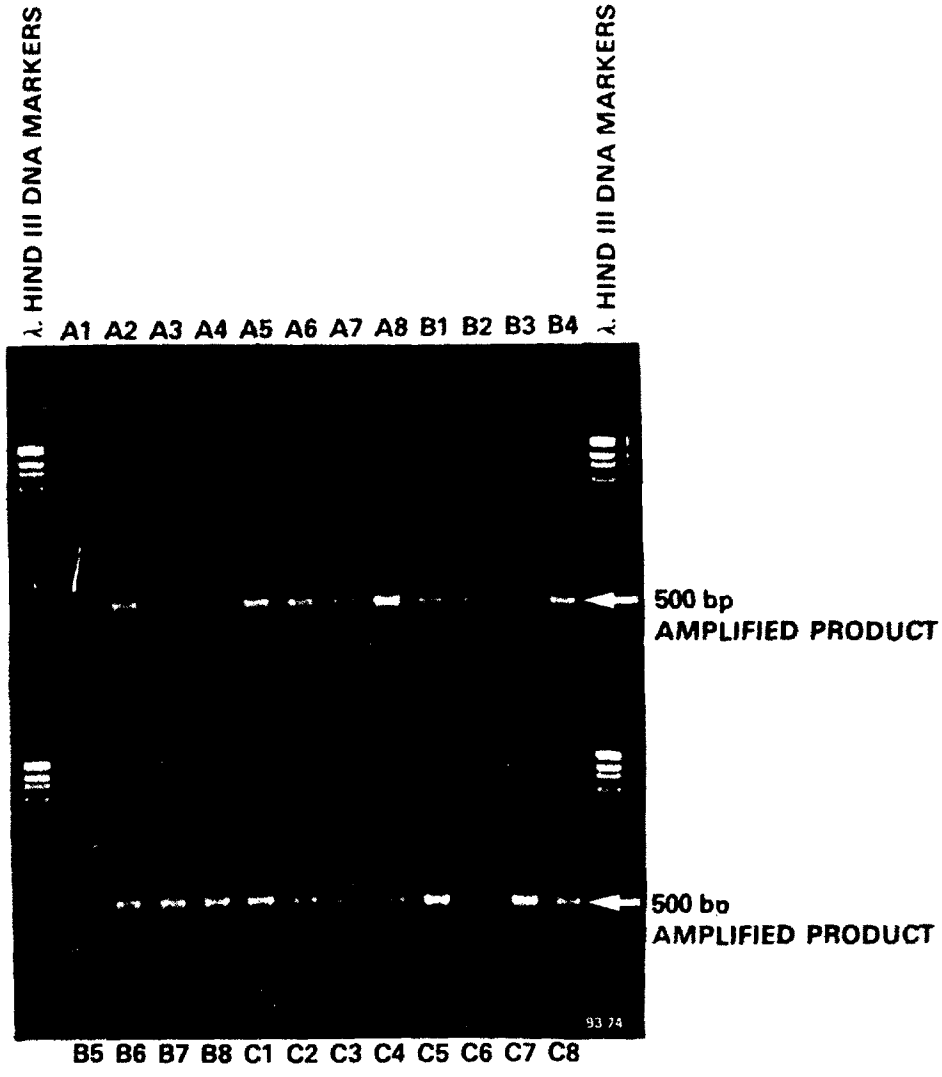


Figure 7a

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 4. Conditions include using 0.5 μL GeneAmp™ microfuge tubes, auto-tune test and heavy oil/94°C start. **Top row** = heating block well positions A1 through B4, **Bottom row** = heating block well positions B5 through C8.

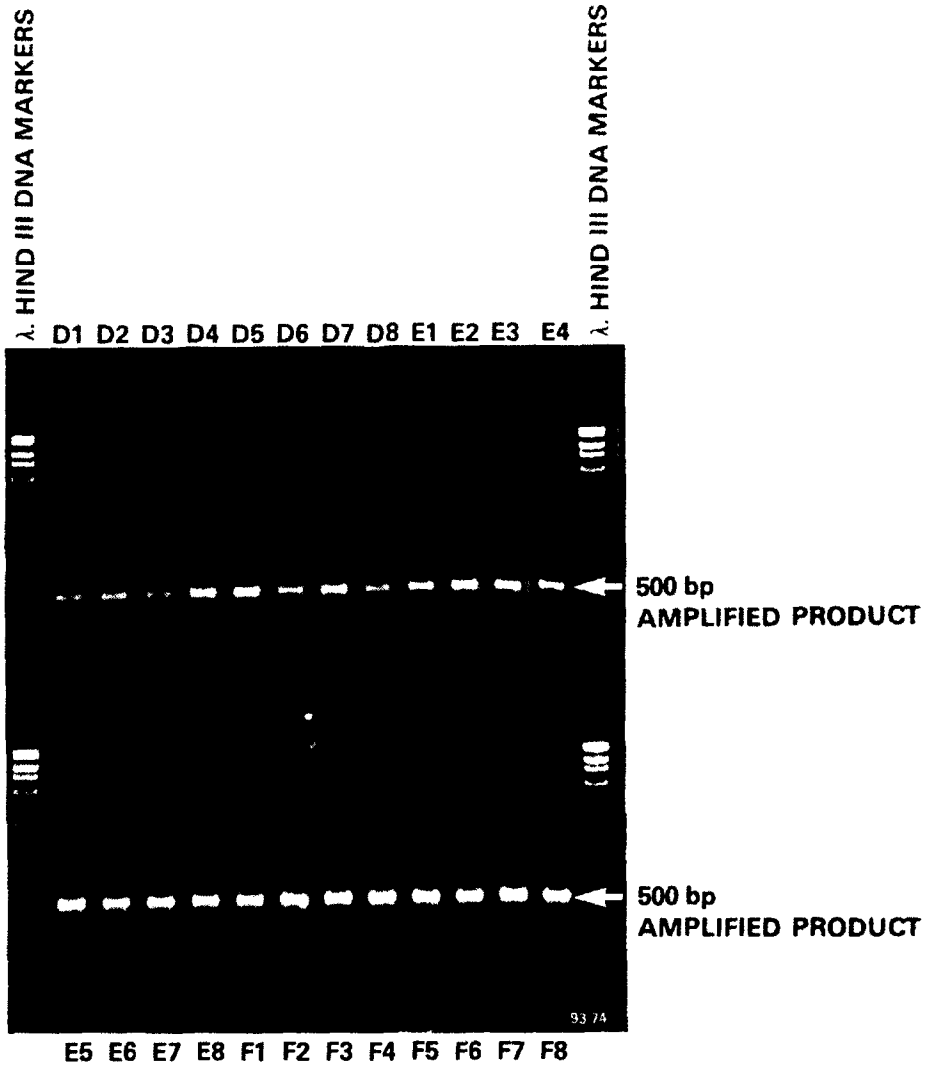


Figure 7b

Horizontal agarose gel analysis of PCR amplified lambda phage DNA from Experiment 4. Same as Figure 7a except: **Top row** = heating block well positions D1 through E4, **Bottom row** = heating block well positions E5 through F8.

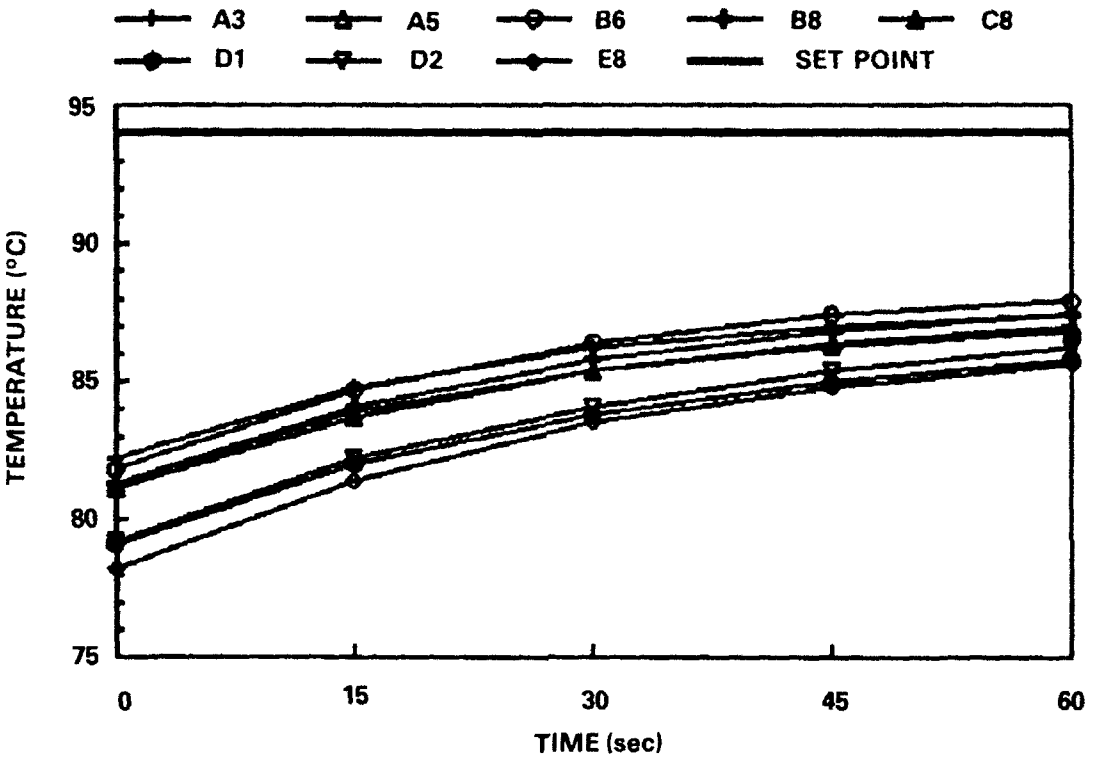


Figure 8

Temperature profile curves for eight well positions of the thermal cycler heating block during a 60 second soak period for the denaturation event (94°C). The temperature readings taken at 0, 15, 30, 45 and 60 sec for each curve are based on the mean of six measurements for a single trial. The setpoint temperature is indicated by the heavy horizontal line.

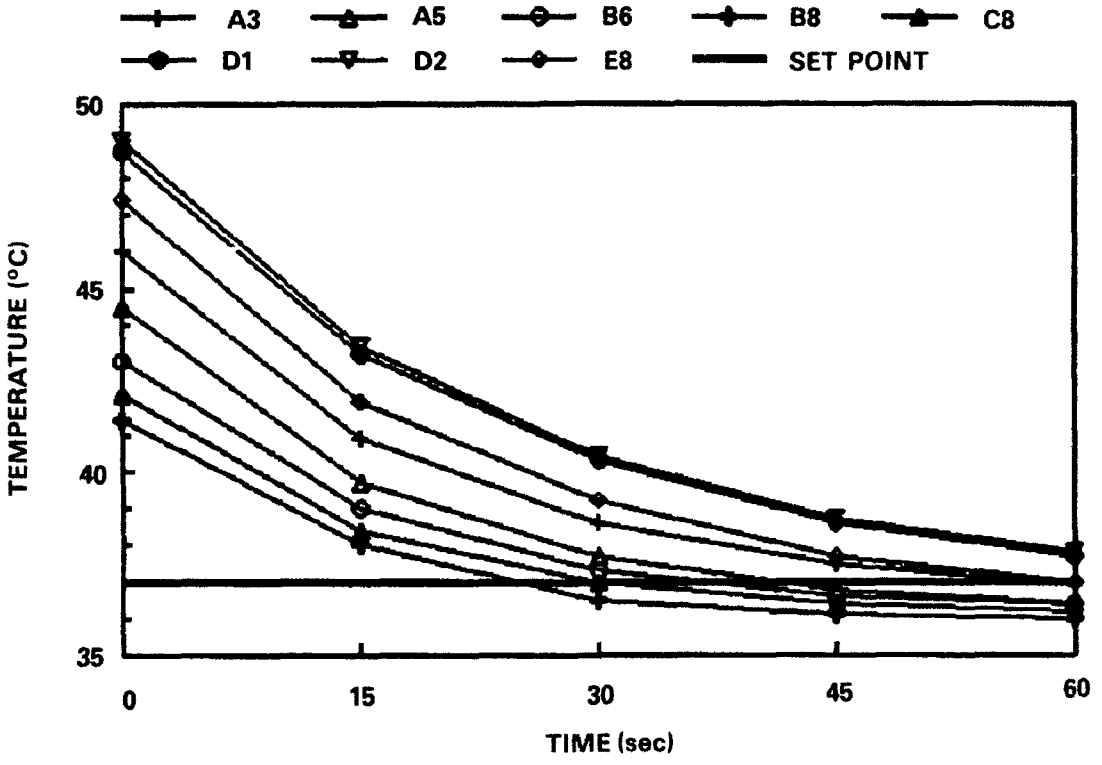


Figure 9

Temperature profile curves for eight well positions of the thermal cycler heating block during a 60 second soak period for the hybridization event (37°C). The temperature readings taken at 0, 15, 30, 45 and 60 sec for each curve are based on the mean of six measurements for a single trial. The setpoint temperature is indicated by the heavy horizontal line.

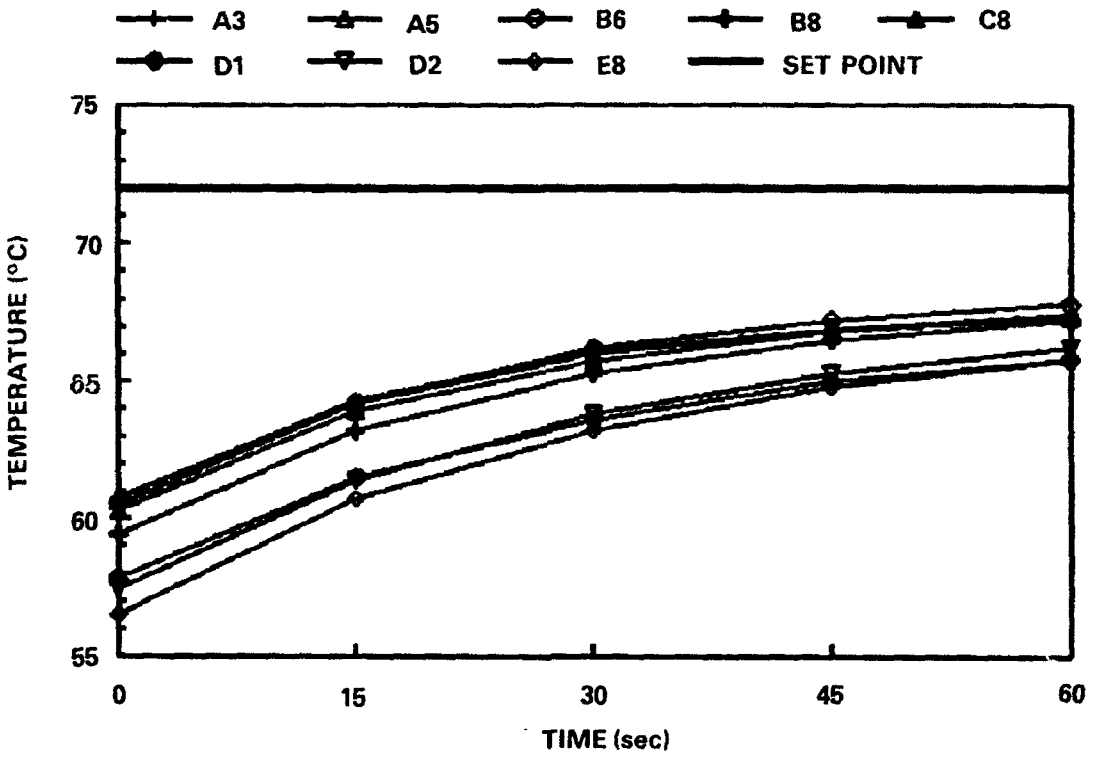


Figure 10

Temperature profile curves for eight well positions of the thermal cycler heating block during a 60 second soak period for the extension event (72°C). The temperature readings taken at 0, 15, 30, 45 and 60 sec for each curve are based on the mean of six measurements for a single trial. The setpoint temperature is indicated by the heavy horizontal line.

ANNEX A-1

PERFORMANCE COMPARISON MATRIX

	CETUS PERKIN-ELMER	ERICOMP	TECHNE	TYLER RESEARCH
USER INTERFACE[1]	2 x 40 Display	2 x 40 Display	4 x 40 Display	25 x 80 Computer
STANDARD SAMPLE[2]	48 - 0.5ml tube	29 - 1.5ml tube	54 - 0.5ml tube	88 - 0.5ml tube
RAMPING FUNCTION[3]	Programmable	No (2 rates)	Limited (4 rates)	Programmable
RAMPING RANGE[4]	0 - 0.2°C/sec	0.09°C/sec max.	0.3°C/sec max.	0 - 0.5°C/sec
HOMOGENEITY[5] DURING RAMP	±5°C	±10°C	±7°C	±1°C
HOMOGENEITY[6] DURING SOAK	±3°C	±4°C	±5°C	±1°C
SAMPLE SENSOR[7]	NO	YES	YES	YES
GRAPHICS[8]	NO	NO	NO	YES
COUNTRY OF ORIGIN	U.S.A.	U.S.A.	U.S.A.	CANADA

[1] This is the means by which the user programs the instrument and is given information relating to the run while it is in progress. Two- and four-line displays show limited information at any one time, necessitating repeated "stepping through" of the programming functions to review the programmed parameters. Because they make use of a full 25-line by 80-character computer screen, Tyler Research Instruments is able to display all programming and operating information on a single screen at one time, thereby simplifying programming and analysis functions.

[2] This is the configuration normally supplied with the instrument. Some manufacturers offer alternate sample formats, such as microtiter plates. The Tyler Research reactor has the largest array of alternative trays available, accomodating 0.5ml and 1.5ml microfuge tubes, microtiter trays, and vertical and horizontal *in situ* slide trays, with other sample formats currently under development.

[3] Ramping refers to the controlled move from one temperature to another. In the two instruments with fully programmable ramping parameters (Cetus/Perkin-Elmer and Tyler Research), the range of ramp rates is from 0°C per second to the maximum average attainable by the instrument under a variety of ramping conditions. In the Ericomp there are two possible ramp rates, corresponding to slow and extremely slow. The Techne, while much faster than the Ericomp, has only 4 available ramp rates which are not programmable by the user. In both cases, the number shown represents the maximum available ramp rate.

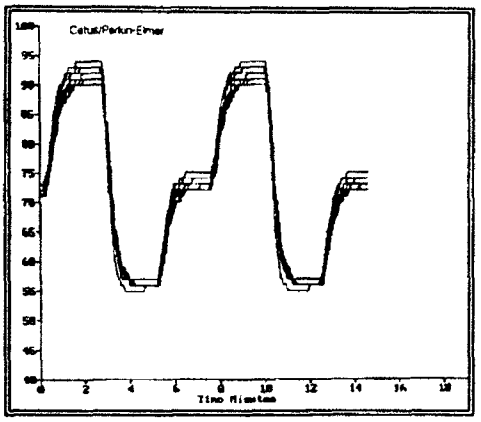
[4,5,6] In each case, figures are those obtained during actual tests conducted in the Faculty of Medicine at the University of Alberta, following manufacturer's instructions for each instrument. A computer-controlled solid state array consisting of 8 laser-trimmed sensors placed inside individual tubes was monitored throughout programmed runs set to a two-minute 92°C soak, a rapid ramp to 55°C for a two-minute soak, a rapid ramp to 72°C for a two minute soak, and a repeat of this cycle. Complete test results including graphic output of all runs for each instrument are available upon request, and representative graphs are presented overleaf. Homogeneity in the Cetus/Perkin-Elmer, Ericomp, and Techne cyclers is poor, particularly in light of the advertised figures. Claims of ±0.1° to ±0.5°C are typical for these manufacturers. Maximum ramp rates are also grossly inflated in their specifications. The Cetus PCR cycler, for example, advertises rates of 1°C per second, although the maximum obtained in the University of Alberta tests was 0.3°C per second, with an average under all test conditions of only about 0.2°C per second. The Tyler Research Instruments thermal reactor is the only device tested that met advertised specifications.

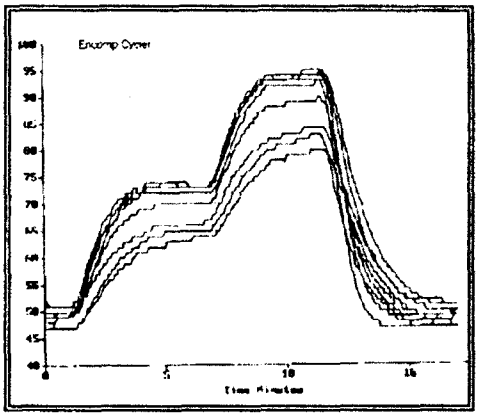
[7] The Cetus/Perkin-Elmer PCR does not have an encapsulated temperature sensor intended to mimic the conditions of the actual samples. Instead, this manufacturer apparently makes the assumption that monitoring the temperature of the block in which the samples are placed is adequate. The University of Alberta results clearly indicate that this is not an accurate assumption. The Ericomp and Techne incorporate an epoxy-imbedded temperature sensor which occupies a position in the temperature block. The Tyler Research instrument uses a tube identical to the sample tubes themselves, to yield constant feedback on sample temperatures. This information is interpreted by the computer controlling the instrument to provide fine control of actual sample conditions.

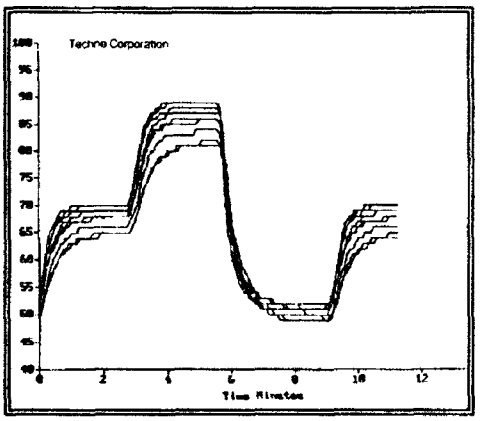
[8] Only the Tyler Research reactor has built-in graphics functions to permit detailed graphic output of all run conditions, including reactor chamber temperature, actual sample temperature, and programmed temperatures. All may be plotted on the computer screen with respect to time, and both graph axes are fully definable by the user. The information is stored digitally for on-line or subsequent analysis and graphs may be printed on a variety of output devices, including dot-matrix and laser printers. This is particularly important in research applications for fine-tuning of experimental parameters. This function is also crucial for courtroom presentation in the case of forensic evidence, and for medical records in the case of molecular diagnostics applications.

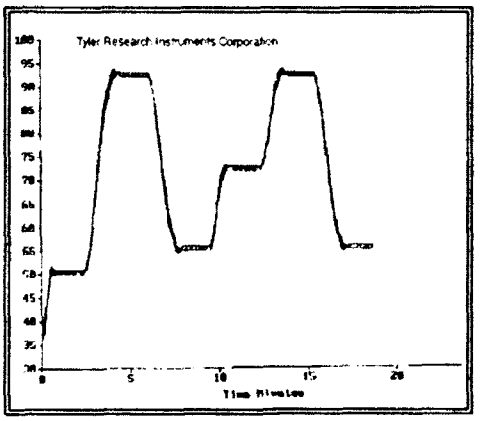
ANNEX A-2

SAMPLE HOMOGENEITY









Representative graphs illustrating sample homogeneity among tubes grouped in a single quadrant of each instrument's sample block. Each graph shows 8 active temperature tracings in real time, plus a single tracing showing the average of the 8 sample tracings. In those cases where fewer than 9 tracings are evident, this is due to concurrence of one or more tracings (reflecting an increased degree of thermal and temporal homogeneity). Data is derived fro n solid-state temperature sensors (AD590JH) immersed in 100µl of mineral oil in sealed microfuge tubes. The sensors (calibrated and matched to within 0.1°C) were sampled at 0.5 second intervals throughout the programmed runs by an IBM microcomputer operating through an 8-channel analog-to-digital board, and all data stored to disk for subsequent graphic analysis.

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Thermal Cycler Temperature Variation and its Effect on the Polymerase Chain Reaction

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Bader, Doug E.

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This study was undertaken to investigate the source of variation in Polymerase Chain Reaction (PCR) amplification assays that we have encountered periodically in our studies. Two approaches, namely, PCR/agarose gel analysis and thermal probe analysis, were used in this investigation. PCR/agarose gel analysis demonstrated random variation in the quantity and quality of amplified product from well to well, both within and between trials. Several modifications to the procedure did not eliminate variability. Thermal probe analysis indicated significant well to well temperature variability among certain wells or groups of wells within a trial ($p=0.01$) and significant temperature variability among certain well positions from one trial to another ($p=0.05$). Thermal probe analysis also indicated large differences between the programmed setpoint temperatures and the actual temperatures inside the tubes for all three PCR events at the beginning of the soak period and two of three PCR events at the end of the soak period. The data from this investigation and other studies leads to the conclusion that the source of variability in PCR amplification efficiency we have experienced is most likely due to the inherent inability of our thermal cycler to maintain consistent temperature homogeneity across the heating block during PCR amplification reactions. It is advisable that replicate samples for amplification be prepared since a particular well cannot be expected to provide consistent results from well to well within a trial or from one trial to the next.

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PCR

Polymerase Chain Reaction

Thermal cycler

DNA amplification

Temperature variability

Amplification variability